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Interleukin-8: A pathogenetic role in antineutrophil cytoplasmic autoantibody-associated glomerulonephritis

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Interleukin-8: A pathogenetic role in antineutrophil cytoplasmic autoantibody-associated glomerulonephritis.

Background. In neutrophil trafficking, the role of interleukin-8 (IL-8) is location dependent. Tissue IL-8 directs transmigration, whereas intravascular IL-8 frustrates this process. The bystander damage of glomerular endothelium by antineutrophil cytoplasmic autoantibody (ANCA)-activated neutrophils is believed to be an early event in the pathogenesis of ANCA-associated glomerulonephritis. We have studied the role of IL-8 in this process.

Methods. Intraglomerular expression of IL-8 in patients with ANCA-associated glomerulonephritis was studied by *in situ* hybridization and immunohistochemistry and location of neutrophils by serial section immunohistochemistry. *In vitro*, we analyzed ANCA-stimulated neutrophil IL-8 production by enzyme-linked immunosorbent assay, and the IL-8 attributable effect of ANCA-stimulated neutrophil supernatant by chemotactic and transendothelial assays.

Results. There was intraglomerular expression of IL-8 at segmental, crescentic, and parietal epithelial sites. IL-8 protein expression colocalized to intraglomerular neutrophils; many localized within glomerular capillary loops, suggesting failed trafficking to tissue IL-8. ANCAs differentially stimulated time- and dose-dependent neutrophil IL-8 production, and ANCA-stimulated neutrophil supernatant demonstrated potent IL-8–dependent chemotactic activity and inhibited transendothelial migration of normal human neutrophils toward an IL-8 gradient.

Conclusion. Despite heavy tissue expression of IL-8 in ANCA-associated GN, the production of IL-8 by ANCA-stimulated neutrophils within the intravascular compartment may frustrate neutrophil transmigration, encourage intravascular stasis, and contribute to bystander damage of glomerular endothelial cells.

The CXC chemokine interleukin-8 (IL-8) is a potent neutrophil chemoattractant [1] and also stimulates neutrophils to degranulate and produce reactive oxygen species [2]. The tissue production of IL-8 by activated resident cells and infiltrating leukocytes at sites of immune and inflammatory injury directs neutrophil infiltration [3]. Conversely, the ligation of circulating neutrophils by IL-8 within the intravascular compartment frustrates this process. The intravascular administration of IL-8 causes sequestration of neutrophils within the microvasculature and failed trafficking of intravascular neutrophils to sites of extravascular IL-8 and other neutrophil chemoattractants [4]. The production of a transgenic overexpressing IL-8 mouse provided further insights into this process [5]. These animals have elevated serum IL-8 levels, sequestrate neutrophils within the microvasculature, and demonstrate severe inhibition of neutrophil transmigration to sites of extravascular inflammation.

A major cause of acute renal failure in adults is the primary small vessel vasculitides, Wegener's granulomatosis, and microscopic polyangiitis [6]. The histologic features of these diseases comprise a necrotizing vasculitis of small arteries and veins, arterioles, venules, and capillaries. Within the kidney, the characteristic early lesion is a focal, segmental necrosis of capillary loops that may progress to a frank crescentic glomerulonephritis [7, 8]. At sites of early disease, thrombosis also occurs within the microvasculature in the absence of extravascular leukocyte accumulation [9]. Lyzed neutrophils are often present within these lesions [10].

Autoantibodies against neutrophil cytoplasmic antigens (ANCAs) have an established association with Wegener's granulomatosis and microscopic polyangiitis [11–13]. Indirect immunofluorescence studies on ethanol-fixed neutrophils identify two main patterns of neutrophil binding. The first is granular cytoplasmic staining (C-ANCA); these antibodies are usually present in the sera of patients with Wegener's granulomatosis [14], and their target antigen is the primary neutrophil granule protein, proteinase 3 (PR3). The second is a perinuclear staining pattern (P-ANCA). These antibodies are usually present in the sera of patients with microscopic polyangi-

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itis. Most perinuclear staining patterns are directed against myeloperoxidase (MPO) [15], although some are directed against PR3 and other primary granule proteins such as elastase and lactoferrin.

Antineutrophil cytoplasmic autoantibodies have been implicated in the pathogenesis of primary systemic vasculitis. *In vivo*, ANCA titers correlate with disease activity [16]. *In vitro*, ANCAs activate tumor necrosis factor-α (TNF-α)-primed neutrophils to degranulate and produce reactive oxygen species [17] and proinflammatory cytokines [18]. These studies and the location of activated neutrophils in early disease suggest that the ligation of neutrophils by ANCAs within the microvasculature triggers bystander damage to endothelial cells. This is supported by cytotoxicity assays in which ANCAs activate primed neutrophils to lyze-cultured endothelial cells [19].

To establish a role for IL-8 in ANCA-associated disease, we examined the expression, distribution, and neutrophil colocalization of IL-8 mRNA and protein in human ANCA-associated glomerulonephritis. We then analyzed the ability of ANCAs to stimulate neutrophils to produce IL-8–rich supernatant, and assessed the biological role of this product in chemotactic and transendothelial assays on normal human donor neutrophils.

METHODS

Tissue collection and preparation

Following local ethical committee permission and informed consent, renal biopsies were obtained from 10 patients with a clinical diagnosis of ANCA-positive glomerulonephritis. For comparison by immunohistochemistry, we analyzed eight renal biopsies from patients with ANCA-negative immune/inflammatory glomerulonephritis: four with lupus nephritis (WHO grade III/IV) and four with Henoch-Schönlein nephritis. Negative control tissue comprised biopsies from patients with minimal change nephropathy ($N = 3$) and cortical fragments from the unaffected pole of kidneys removed for renal cell carcinoma ($N = 5$). Each biopsy was immediately embedded in Brights cryo-m-bed medium (BDH, Poole, Dorset, UK), snap frozen in liquid nitrogen, and stored at −70°C until sectioning. For *in situ* hybridization (ISH) studies, 7 μm cryostat sections were collected onto Superfrost Plus slides (BDH), were immediately fixed in freshly prepared 4% paraformaldehyde in phosphate-buffered saline (PBS; pH 7.4) for two hours at 4°C, were washed briefly (×2) in diethyl pyrocarbonate-treated water, and were vacuum desiccated and stored at −70°C until use. Cryostat sections for immunohistochemistry studies were air dried, fixed in acetone for 10 minutes, and stored at −20°C until use. Where serial acetone-fixed sections were available, colocalization of IL-8 protein and CD15 positive cells (neutrophils) was studied. Unless otherwise indicated, all reagents for ISH and immunohistochemistry studies were obtained from Sigma (Poole, Dorset, UK).

Riboprobe synthesis

Riboprobes were synthesized from a 300 base pair IL-8 complementary DNA fragment (polymerase chain reaction product) ligated into a pCR 11 Vector plasmid at a Pst 1 restriction site. Sense and antisense probes were generated using DNA-dependent SP6 and T7 RNA polymerase promoters (Promega, Southampton, UK) in a transcription reaction incorporating 50 μCi of ^{35}S uridine triphosphate (UTP; Dupont, Belgium). Probes were labeled to a specific activity of 1 to 2×10^8 dpm/μg, purified on RNase-free sephadex G50 columns (Amersham, Little Chalfont, Buckinghamshire, UK), and characterized by polyacrylamide/urea gel electrophoresis alongside transcription generated labeled standards (data not shown). Labeled probe (2×10^7 cpm) was diluted to a volume of 208 μl in 50 μl tRNA (10 mg/ml), 50 μl denatured herring sperm DNA, 10 μl 1 M dithiothreitol, and diethyl pyrocarbonate-treated water, was heated at 65°C for five minutes, was then brought to a volume of 1 ml by hybridization solution [66% formamide, 7.5% dextran sulfate, 1.33 mM ethylenediaminetetraacetic acid (EDTA; pH 8.0), 13.33 mM Tris (pH 8.0), and 0.4 M NaCl], and was kept at 65°C for immediate use. The cDNA plasmid template was a gift from Professor D. Adams and Dr. S. Afford (Liver Laboratories, Queen Elizabeth Medical Center, Birmingham, UK).

In situ hybridization

Sections were brought to room temperature, vacuum desiccated for 30 minutes, and digested in 0.001% proteinase K (in 100 mM Tris, pH 8.0, 50 mM EDTA, pH 8.0) for 30 minutes at 37°C, refixed in 4% paraformaldehyde in PBS for 20 minutes, rinsed briefly (×2) in water, equilibrated in 0.1 M triethanolamine at pH 8.0 for 2.5 minutes, and acetylated in 0.2% acetic anhydride in triethanolamine for 10 minutes at room temperature. Sections were washed briefly in 2 × standard saline citrate (SSC), dehydrated in ascending ethanol concentrations (50 to 100%), and vacuum desiccated for 60 minutes. They were then immediately hybridized with 1×10^6 cpm of probe in a volume of 50 μl for 16 hours at 52.5°C. Post-hybridization, sections were sequentially washed in 4 × SSC (four changes), digested with 20 μg/ml RNase A (in 0.5 M NaCl, 0.01 M Tris, pH 8, 0.001 M EDTA, pH 8.0) for 30 minutes at 37°C, desalinated at room temperature (in descending SSC concentrations), and washed in 0.1 × SSC for 60 minutes at 65°C. Sections were then briefly rinsed in 0.1 × SSC at room temperature and dehydrated in ethanol with 0.1 × SSC. Post-RNase A treatment, dithiothreitol was added at a concentration of 1 mM to all solutions. Dehydrated sections were further vacuum desiccated, dipped in photographic emulsion

(Ilford's K5, Ilford, UK), and stored desiccated at 4°C for three weeks. They were then developed, fixed, and counterstained with hematoxylin and eosin. Controls included the sense probe and RNase A pretreated sections. Positive chemography was excluded by running paralleldipped nonhybridized slides through the reaction sequence.

Immunohistochemistry

All incubations were performed at room temperature in a humidified box. Endogenous peroxidase, avidin, and biotin were blocked with serial 10-minute incubations of 0.1% azide/0.3% H_2O_2, 0.1% avidin, and 0.01% biotin. Following a 10-minute blocking stage with 20% normal rabbit serum, three-stage indirect immunohistochemistry was performed with primary mouse antihuman IL-8 (a gift from Sandoz, Geneva, Switzerland) and CD15 (Dako, Glostrup, Denmark) monoclonal antibodies, biotin-labeled rabbit antimouse secondary antibodies, and a streptavidin/ABC/HRP (Dako) conjugate. To confirm the location of neutrophils, four disease and four positive control biopsies were serial stained with a monoclonal antibody (Dako) against the endothelial cell marker von Willebrand factor (vWf). Control sections comprised substitution of the primary antibody stage with Tris-buffered saline. Binding of the tertiary complex was visualized by the addition of diaminobenzidine (3,3′-diaminobenzidine tetrahydrochloride). Tissue was counterstained with hematoxylin, mounted in dibutyl polystyrene xylene (DPX), and coverslipped.

In vitro studies

IgG isolation. Serum was collected from patients with vasculitis who fulfilled the Chapel Hill Consensus Conference definitions for Wegener's granulomatosis or microscopic polyangiitis [20]. PR3 and MPO specificity of patient serum was determined as previously described [21]. ANCA-rich IgG was prepared from patient and normal human donor sera using protein G columns as previously described and concentrated using Vivaspin 15 centrifugal concentrators (Vivascience, Lincoln, UK) [18]. $F(ab')_2$ IgG fragments were obtained by incubating whole IgG with pepsin at 37°C for 16 hours, followed by repeated chromatography on protein G columns. Endotoxin contamination of IgG fractions was monitored using a Limulus amoebocyte assay. Levels were always below 480 pg/ml by this assay. Immune complexes were removed by centrifugation at 30,000 × g for 10 minutes. The purity of both $F(ab')_2$ and IgG fragments was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and the total protein content was determined by spectrophotometry [18]. $F(ab')_2$ fragments were still able to bind to alcohol-fixed neutrophils, as shown by indirect immunofluorescence. Further, an enzyme-linked immunosorbent assay (ELISA) using PR3-coated wells (Binding Site, Birmingham, UK) was used to compare the binding activity of anti-PR3 whole IgG and $F(ab')_2$. The plates were blocked as described later here (for IL-8) and a range of IgG and $F(ab')_2$ concentrations was added to the wells and incubated for two hours at 37°C. Following a further incubation of one hour with a monoclonal antihuman IgG F(ab) peroxidase conjugate at a 1:1600 dilution, the reaction was developed, and absorbance was measured as described earlier here. Some additional studies were undertaken using a mouse monoclonal anti-PR3 antibody, 4A5 (a gift from Dr. J. Weislander, Lund, Sweden), and isotype-matched control mouse IgG (Dako).

Neutrophil isolation and stimulation. Neutrophils were isolated by density gradient centrifugation across percoll and were resuspended at 1×10^7 cells per ml in Roswell Park Memorial Institute (RPMI) medium supplemented with 2.2 mM glutamine [18]. Neutrophils were 99% viable by trypan blue exclusion and were 98 to 99% pure by hematoxylin staining. Neutrophils were primed for five minutes with 5 μg/ml cytochalasin B (Sigma) followed by 80 U/ml TNF-α (NIBSC, Potters Bar, UK) for a further 15 minutes and were plated onto 96-well tissue culture plates (Falcon, Oxford, UK) at a density of 5×10^5 cells per well. The cells were treated with 200 μg/ml (unless otherwise stated) of ANCA IgG, normal IgG, or $F(ab')_2$ and were incubated at 37°C with 5% CO_2 for six hours (unless otherwise stated).

ELISA. Neutrophil supernatants were isolated by centrifugation at 11,000 × g for two minutes and were assayed in triplicate for IL-8. Ninety-six–well plates (Nunc, Paisley, UK) were coated overnight (at 4°C) with 100 μl of 1.25 μg/ml monoclonal IL-8 antibody (R&D, Abingdon, UK) in a carbonate buffer (0.03 M Na_2CO_3, pH 9.6). The plate was blocked for one hour with 10% dried skimmed-milk powder in PBS (200 μl per well), and supernatant was added at 100 μl per well. Recombinant human IL-8 (R&D) was used at concentrations ranging from 16 pg/ml to 1000 pg/ml to standardize the ELISA and 100 μl added per well. The standards were diluted in PBS, 0.5% bovine serum albumin, and 0.05% Tween 20. After one hour, 100 μl of goat polyclonal anti-human IL-8 antibody (R&D) diluted in PBS containing 10% dried skimmed-milk powder was added at a concentration of 2 μg/ml. After one further hour, a peroxidase-conjugated donkey antigoat IgG (Binding Site) diluted in PBS containing 10% dried skimmed-milk powder was added at a 1 in 1000 dilution. After a one-hour incubation, 5 μl of 30% H_2O_2 was added to 25 ml of 0.4 mg/ml o-phenylenediamine hydrochloride in citrate buffer (0.1 M citric acid solution made up to pH 5.0 using K_2HPO_4), which was then added to the plate. The reaction was stopped after 15 minutes using 20% H_2SO_4 (50 μl per well), and the absorbance was measured at 492 nm using a Multiskan bichromatic analyzer (Lab-

systems, Helsinki, Finland). The ELISA was capable of detecting 20 pg/ml of IL-8, and variation between tests was less than 10%. The assay was performed at 37°C until the addition of H_2O_2, when subsequent steps were performed at room temperature.

Chemotactic assay. An agarose chemotactic assay was a modification of methods previously described [22]. Briefly, 6 ml of RPMI (Sigma) supplemented with 2.5% gelatin, 2% fetal calf serum (First Link, Brierley Hill, UK), and 1% agarose were poured into 5 cm Petri dishes. Three equidistant wells were cut into the agar with a 3.2 mm diameter punch, and neutrophils (1×10^7 per ml) were added to the center wells. RPMI was added to the inner well and neutrophil supernatant (prepared as described), or 1000 pg/ml IL-8 was added to the outer well. Plates were incubated at 37°C for two hours in the presence of 5% CO_2. After two hours, the plates were fixed for 30 minutes in 2.5% glutaraldehyde (Sigma) and were stained with crystal violet (Sigma). The chemotactic response was determined using an eyepiece micrometer graticule (Graticules Ltd., Tonbridge, UK) by calculating the chemotactic index (chemotactic minus chemokinetic distance) for each well. Chemotactic distance is the distance covered by neutrophils toward a chemoattractant stimulus. Chemokinetic distance is the distance covered by neutrophils by random kinetic movement in any direction, measured here away from the chemoattractant stimulus.

Endothelial cell transmigration assay. Endothelial cells were isolated from human umbilical cord veins according to methods described previously [23] and were used at passage two or three. The cells were plated at confluent density (3×10^5 cells per insert) onto gelatin-coated cell culture inserts (3 μm pore size) positioned in 24-well tissue culture plates (Falcon, Oxford, UK). After overnight culture, the cells were washed, and inserts were transferred to a 24-well plate to which 50 ng/ml IL-8 in RPMI supplemented with 2.5% fetal calf serum and 2.2 mM glutamine had been added to each well (lower chamber). Neutrophils were incubated for 30 minutes with neutrophil supernatant from ANCA-stimulating experiments, supplemented with 2.5% fetal calf serum, and seeded onto the inserts at 1.5×10^6 cells per well. The plates were incubated for 45 minutes at 37°C with 5% carbon dioxide. After 45 minutes, the inserts were removed, and the underside was rinsed with PBS. Neutrophil migration across the endothelium-covered inserts into the lower chamber was determined by direct counting using a hemocytometer chamber. In preliminary studies, neutrophils were shown to demonstrate increasing levels of transmigration in response to increasing concentrations of IL-8 in the lower chamber (0 to 1000 pg/ml). The concentration of IL-8 causing half maximum migration was 50 ng/ml, and this was used in the described studies. To assess whether decreased neutrophil transmigration was due to IL-8 in the upper chamber, neutrophil supernatant (350 μl), which had been preincubated for five minutes with 50 μg of polyclonal antihuman IL-8 antibody, was incubated with 300 μl of immobilized protein G (Sigma) at 4°C for one hour. The amount of polyclonal anti-human IL-8 necessary was determined in preliminary experiments to be able to remove all IL-8 present to a concentration of 1000 pg/ml. The supernatant was removed by centrifugation at 12,000 *g* for two minutes, and IL-8 was quantitated by ELISA prior to use of the supernatant in a transmigration assay.

Data analysis

Data is presented as mean and standard error of the mean (SEM) unless otherwise stated. Statistical analysis was by Student's *t*-test, and Pearson's test was used to correlate IL-8 protein expression with presence of neutrophils.

RESULTS

In situ hybridization

The presence of IL-8 mRNA in ANCA-associated glomerulonephritis was defined using a graticule counting system (Graticules Ltd.) as one or more 6.25 μm^2 areas that contain hybridization signals that were five times greater than adjacent background. IL-8 mRNA was expressed within 29.8% (± 6.98%) glomeruli/biopsy. When present within glomeruli, IL-8 mRNA expression was invariably focal at sites of inflammation and infiltrates within glomeruli. Specific expression was by glomerular endothelial and tuft infiltrating cells (Fig. 1 A, C) with further heavy expression in cellular crescents (Fig. 1D) and occasional expression by parietal epithelium (Fig. 1D). Within the glomerulus, macrophages are the major infiltrating cell, and other studies indicate that these cells are a potent source of IL-8 [1]. Expression was strongest with untreated patients or those patients with relapsing disease. There was also expression local to leukocyte infiltrates at interstitial and vascular sites and by proximal tubular epithelium (Fig. 1E). At some sites (Fig. 1C), intravascular leukocytes were local to the site of IL-8 mRNA production, suggesting that leukocytes themselves may release chemokines in the process of trafficking. Only background levels of grains were detected at tissue edges, in vascular and tubular lumen, and on sense hybridized sections (Fig. 1 B, F). There was minimal positive chemography when the probe was omitted from the reaction sequence. In normal tissue and non-inflammatory glomerulonephritis, there was no glomerular expression and minimal tubulointerstitial expression of IL-8 mRNA.

Immunohistochemistry

Interleukin-8. In ANCA-associated glomerulonephritis, there was a presence of IL-8 protein in 50.28% (± 9.1%)

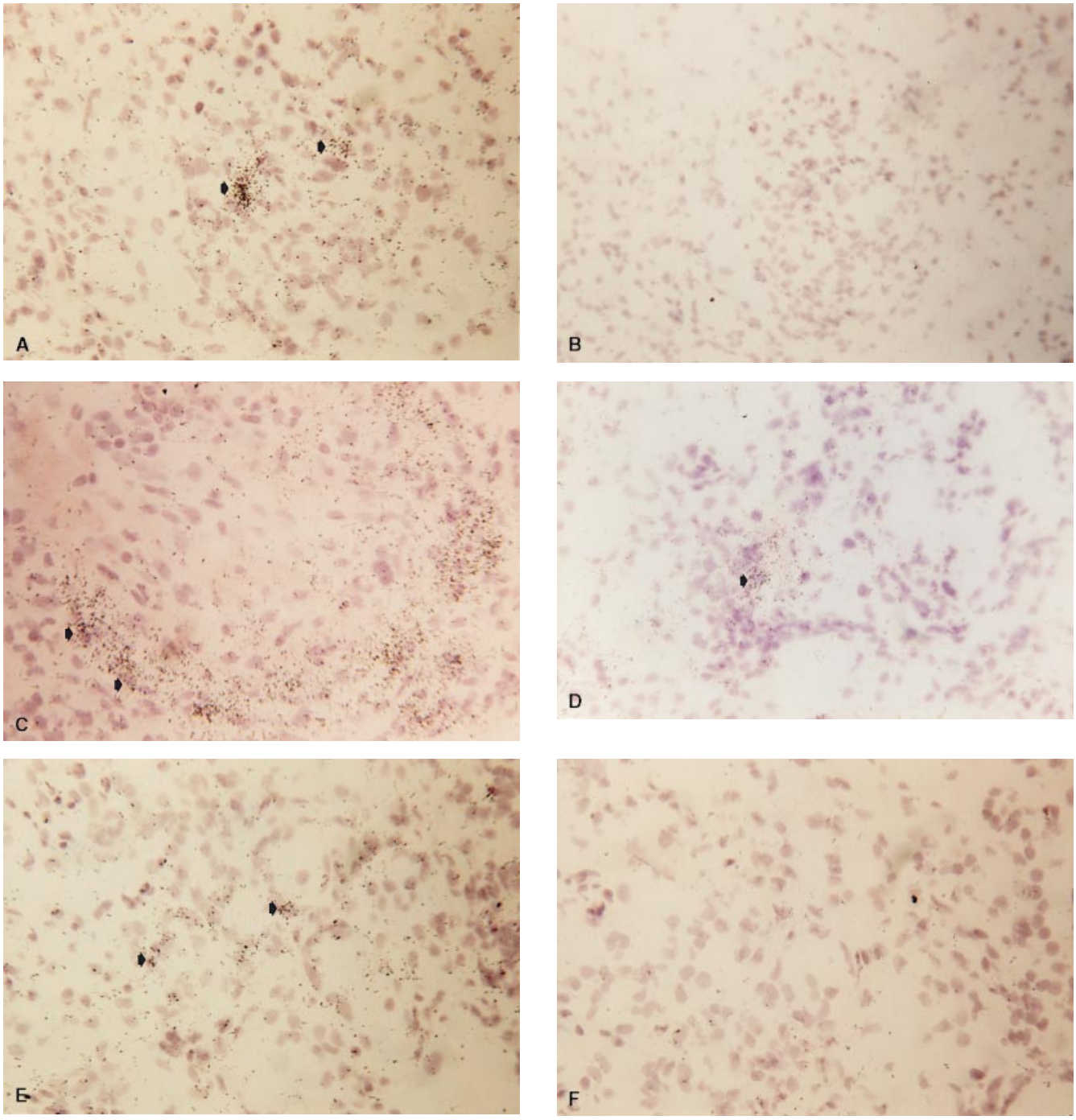

Fig. 1. Interleukin (IL)-8 mRNA expression in antineutrophil cytoplasmic autoantibody (ANCA)-associated glomerulonephritis. (*A*) Intraglomerular expression. Note the localization of IL-8 mRNA (arrows) to glomerular capillary loops (magnification ×200). (*B*) Sense control to panel A. (*C*) Intraglomerular expression of IL-8 mRNA by cells within a crescent and by parietal epithelial cells (arrows; ×320). (*D*) IL-8 mRNA at the site of a segmental lesion within a glomerulus. Note the presence of IL-8 mRNA (arrow) local to a leukocyte within an area of capillary loop thrombosis (×525). (*E*) IL-8 mRNA expression (arrows) by proximal tubular epithelium (×200). (*F*) Sense control to Panel E.

of glomeruli. By ISH, there was a lower proportion of glomeruli positive for IL-8 (mentioned earlier here); however, chemokine mRNA has a short half-life, and the presence within tissue may be relatively short; conversely, chemokine protein may be retained at tissue sites for longer periods of time. IL-8 protein was present in similar patterns to IL-8 mRNA, with intense immunoreactivity at crescentic sites and local to parietal epithelium (Fig. 2A). In addition, there was heavy expression present at segmental sites local to glomerular capillary loops.

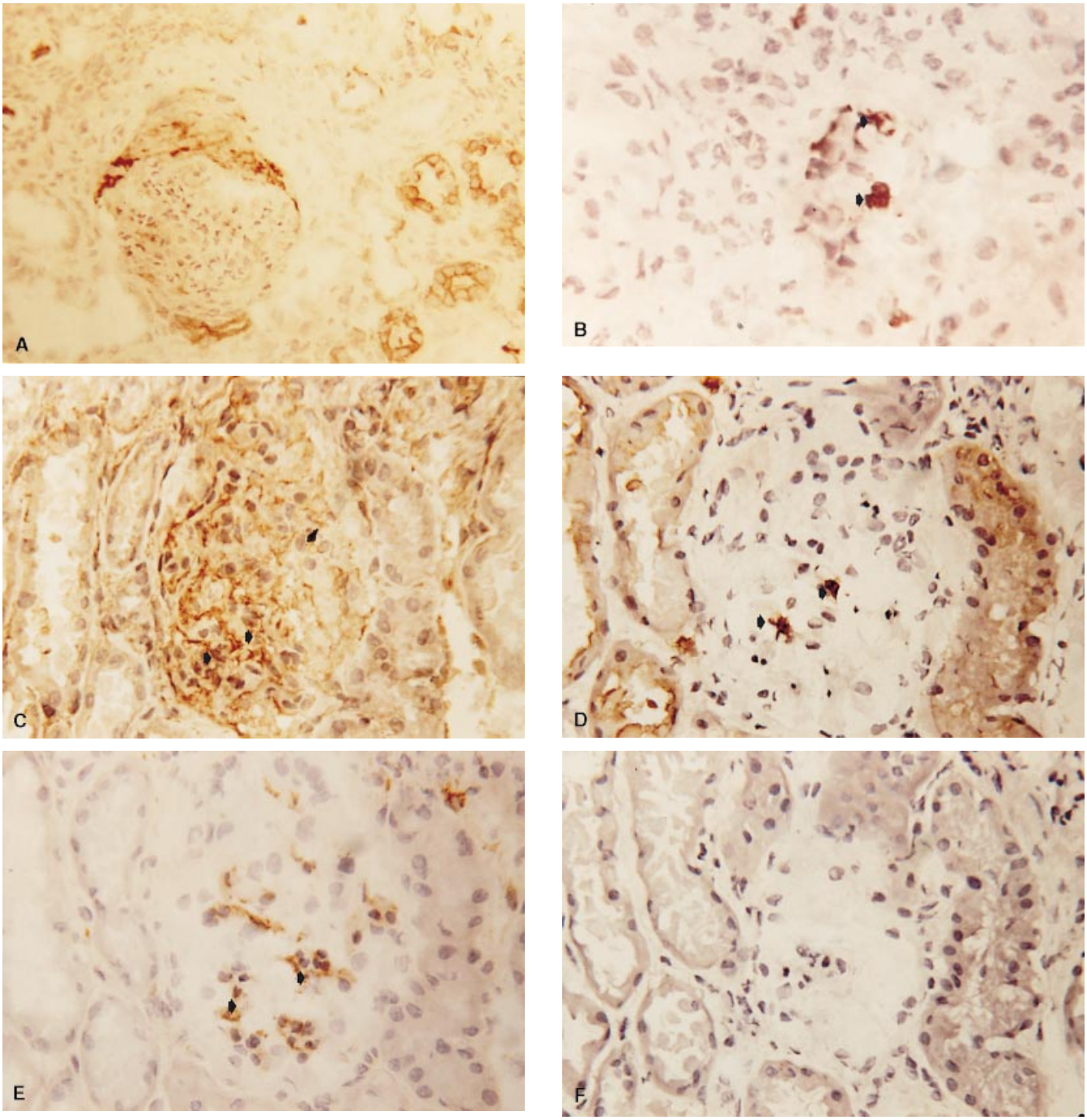

Fig. 2. IL-8 protein expression and neutrophil colocalization in ANCA-associated glomerulonephritis. (*A*) Intraglomerular expression of IL-8 protein within a crescent. Also note parietal epithelial and tubular epithelial expression (magnification ×325). (*B*) Neutrophils (arrows) within capillary loops in a glomerulus in renal biopsy material from a patient with ANCA-associated glomerulonephritis (magnification ×525). (*C*) Heavy intraglomerular IL-8 expression in ANCA-associated glomerulonephritis. The arrows indicate the position of the intravascular neutrophils shown in panel D (magnification ×325). (*D*) Serial section to panel C. Three neutrophils are present within the glomerulus. Two neutrophils (arrows) are localized at and within glomerular capillary loops despite heavy tissue levels of IL-8. Colocalization to panel C indicates that the neutrophils themselves are producing IL-8 (magnification ×325). (*E*) Serial section to panels C and D showing endothelial cells (by vWf). Position of neutrophils is marked by arrows and shows presence within capillary loops and localization adjacent to endothelial cells. (*F*) Control to panels C–E.

Within the tubulointerstitial compartment, there was expression at proximal tubular epithelial (Fig. 2A) and interstitial sites. In ANCA-negative inflammatory glomerulonephritis, there were similar patterns of IL-8 expression with the presence of IL-8 within 60.4% (± 20.3%) of glomeruli. In noninflammatory glomerulonephritis,

Table 1. Intraglomerular neutrophils in glomerulonephritis

	Glomeruli/ biopsy	Neutrophils/ glomerulus	Local to capillary loops	At extra-vascular sites
			%	
ANCA-positive glomerulonephritis	6.57 ± 2.28	3.04 ± 1.35	57.85 ± 42.8	42.15 ± 27.23
ANCA-negative glomerulonephritis	5.11 ± 1.07	2.43 ± 1.41	37.6 ± 23.45	62.4 ± 35.65
Negative control tissue[a]	9.73 ± 7.87	0.64 ± 0.29		

Data show mean ± SEM.
[a] Analysis of site of neutrophils not performed due to sparse numbers

there was occasional tubular expression and, in normal tissue, very occasional glomerular presence. The presence of IL-8 protein correlated with intraglomerular neutrophils in both ANCA-positive ($r = 0.83$, $P < 0.05$) and ANCA-negative glomerulonephritis ($r = 0.65$, $P < 0.05$).

CD15. In ANCA-associated glomerulonephritis, neutrophils were present both within the tubulointerstitium and at glomerular sites. Glomerular presence was sparse compared with infiltrating mononuclear cell types. Most glomerular neutrophils were present within the glomerular tuft and often appeared localized within the glomerular microvasculature (Fig. 2B and Table 1). This was confirmed by serial section analysis on four biopsies. Using anti-vWf antibodies as an endothelial cell marker, these neutrophils were demonstrated to be present within capillary loops and adjacent to endothelial cells. Furthermore, these neutrophils colocalized to glomeruli that were rich in extravascular IL-8, and at some sites, neutrophils themselves expressed IL-8 protein (Fig. 2 C–F). In ANCA-negative inflammatory glomerulonephritis, this approach showed that only a minority of neutrophils were present at intravascular sites (Table 1).

In vitro studies

ANCA induction of neutrophil IL-8: Both anti-PR3 and anti-MPO ANCA stimulated cytochalasin and TNF-α-primed neutrophils to produce IL-8. This was a dose-dependent effect maximal at 200 μg/ml ANCA IgG (Fig. 3). In a time course experiment (performed in duplicate) with anti-MPO ANCAs (at 200 μg/ml) and harvesting of neutrophil supernatant at two-hour time points (to 10 hr), there was a maximal stimulation of IL-8 production at four to eight hours. When compared with normal IgG-stimulated neutrophils, there was significant IL-8 production at each time point from four hours ($P < 0.05$). In a separate experiment (performed in triplicate), the effects of a panel of 10 ANCA IgGs from patients with vasculitis (5 anti-PR3, 5 anti-MPO) were tested by harvesting neutrophil supernatant after six hours of stimulation. All ANCA IgGs demonstrated a consistent but differential ability to stimulate neutrophil IL-8 production when compared with unstimulated neutrophils ($P \leq 0.082$) and normal IgG stimulated neutrophils ($P \leq 0.03$.) A mouse monoclonal antibody to PR3 (4A5) also stimulated dose-dependent production. Control mouse IgG (isotype matched) was nonstimulating.

Inhibition by cycloheximide and actinomycin of anti-MPO ANCA induced IL-8 secretion. Having shown the ability of ANCAs to stimulate neutrophil IL-8 production, the effects of cycloheximide and actinomycin (added to neutrophils immediately before the addition of ANCAs) were examined in an experiment (performed in triplicate) with anti-MPO ANCAs. Neutrophils treated with normal human IgG produced 48 ± 5 pg/ml IL-8. Six-hour stimulation with ANCAs resulted in 121 ± 19 pg/ml IL-8. This response was reduced to 74 ± 5 pg/ml with 2×10^{-5} M actinomycin ($P = 0.028$) and to 51 ± 2 pg/ml with 1×10^{-5} M cycloheximide ($P = 0.03$). There was no significant inhibition of unstimulated neutrophil IL-8 production by actinomycin or cycloheximide. Normal IgG was nonstimulating.

Studies with ANCA F(ab′)$_2$. It has previously been suggested that the engagement of FcγRII receptors is necessary for ANCA stimulation of superoxide release from neutrophils (Fig. 4). In experiments (performed in triplicate) with single IgGs with anti-MPO and anti-PR3 specificities, F(ab′)$_2$ fragments failed to generate neutrophil IL-8 production, suggesting a requirement for Fc receptor engagement for ANCA-stimulated IL-8 production. For anti-PR3 ANCAs, an ELISA against immobilized PR3 was used to compare the ability of ANCA-IgG and F(ab′)$_2$ fragments with similar binding activity to generate neutrophil IL-8 production.

Chemotactic data. Using single anti-MPO and anti-PR3 ANCA-rich IgGs, ANCA-stimulated neutrophil supernatant was biologically active in an agar assay (single experiments performed in triplicate; Fig. 5A). Normal IgG and untreated neutrophil supernatant were minimally stimulating. Neutrophil chemotaxis provoked by anti-MPO and anti-PR3 ANCA supernatant was blocked to 74.5% and 88.8%, respectively, by a blocking polyclonal antibody, indicating that a high proportion of the chemotactic bioactivity of neutrophil supernatant was IL-8 attributable. An isotype-matched control IgG did not abrogate IL-8–attributable chemotaxis and demonstrated the specificity of the blocking antibody.

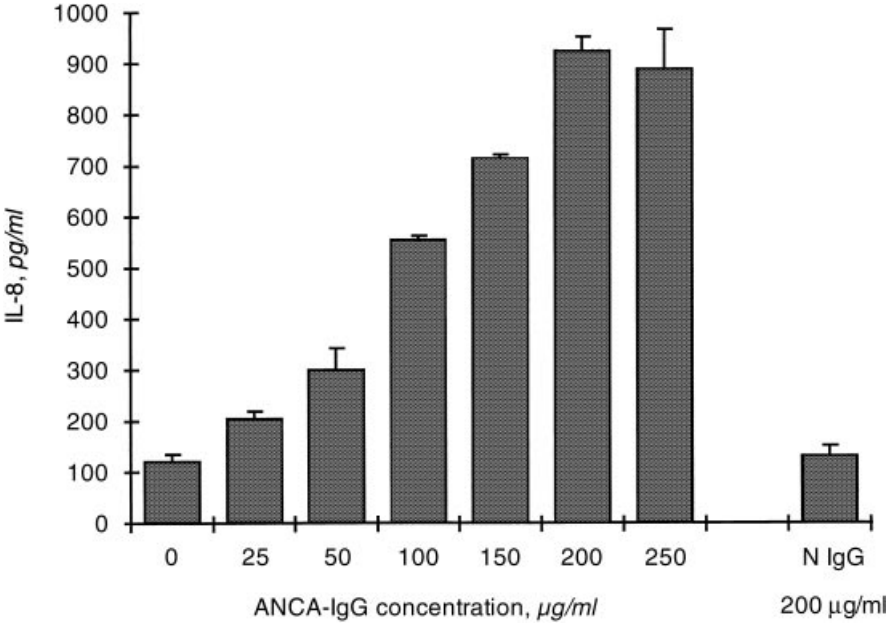

Fig. 3. Dose-response graph showing maximal stimulation of neutrophil interleukin-8 (IL-8) production by anti-myeloperoxidase (MPO) anti-neutrophil cytoplasmic autoantibody (ANCA) at a concentration of 200 μg/ml. Neutrophil supernatant was harvested at six hours. Data show the mean and standard deviations of duplicates of a single experiment. Control (ANCA-negative) IgG was nonstimulating.

Transendothelial transmigration. It has been suggested that intravascular IL-8 prevents neutrophil transmigration. Preliminary transmigration studies confirmed the ability of IL-8 in an upper chamber to inhibit transmigration across an endothelial cell monolayer to a higher concentration of IL-8 in a lower chamber. In transmigration assays with anti-PR3–stimulated neutrophil supernatant, there was inhibition of neutrophil transmigration down a 50 ng/ml IL-8 gradient (Fig. 5B). The IL-8 attributable chemotaxis of this supernatant was confirmed by binding and preabsorption of IL-8. There was no inhibition by normal IgG-stimulated neutrophil supernatant above background.

DISCUSSION

This is the first study, to our knowledge, to identify the expression of IL-8 mRNA and protein *in situ* in ANCA-associated glomerulonephritis, and to investigate a possible pathogenetic role for this molecule in small vessel vasculitis. The heavy expression of IL-8 at sites of glomerular and tubulointerstitial inflammation indicates the *in vivo* presence of local chemoattractant machinery necessary to direct neutrophil trafficking to extravascular sites. This intrarenal expression is consistent with the results of studies on renal cells *in vitro.* Both isolated cultured mesangial cells and proximal tubular epithelial cells produce IL-8 on stimulation by proinflammatory stimuli such as lipopolysaccharide, IL-1 and TNF-α [24, 25]. IL-1 and TNF-α also induce endothelial cells to synthesize IL-8 [26]. Further, in ANCA-associated vasculitis, ISH analysis has demonstrated the expression of the proinflammatory cytokines TNF-α and IL-1β by resident and infiltrating cells [27]. Thus, local stimuli are present *in vivo* that trigger IL-8 production *in vitro.*

Despite our demonstration of heavy intrarenal expression of IL-8 in acute vasculitis, we found rather poor levels of neutrophil infiltration into tissues. Over half of the neutrophils were located at or within glomerular capillary loops. This was consistent across all biopsies studied, suggesting it was not an artifact of examining a single kidney at one point in time. Further, these observations are consistent with the study of Brouwer et al who demonstrated a maximum number of 7 ± 6.4 neutrophils/glomerular cross-section in a subset of severe ANCA-associated glomerulonephritis. Although localization within the glomerulus was not precise, immunofluorescent analysis indicated that some neutrophils were present within the segmental capillary lesions themselves [28]. Neutrophils are sometimes seen close to breaks in the basement membrane of glomerular capillaries [29], both at sites where endothelial cells are intact and at areas of endothelial cell lysis and detachment. At other sites, the presence of lyzed neutrophils within affected vessels in Wegener's granulomatosis has been identified [10].

This evidence for presence of neutrophils local to affected capillary loops, but with poor neutrophil penetration into the IL-8–rich inflamed tissues themselves, led us to consider whether mechanisms might exist that hinder neutrophil trafficking in vasculitis. Vasculitis is associated with ANCAs that may activate neutrophils, leading to the release of proinflammatory mediators such as reac-

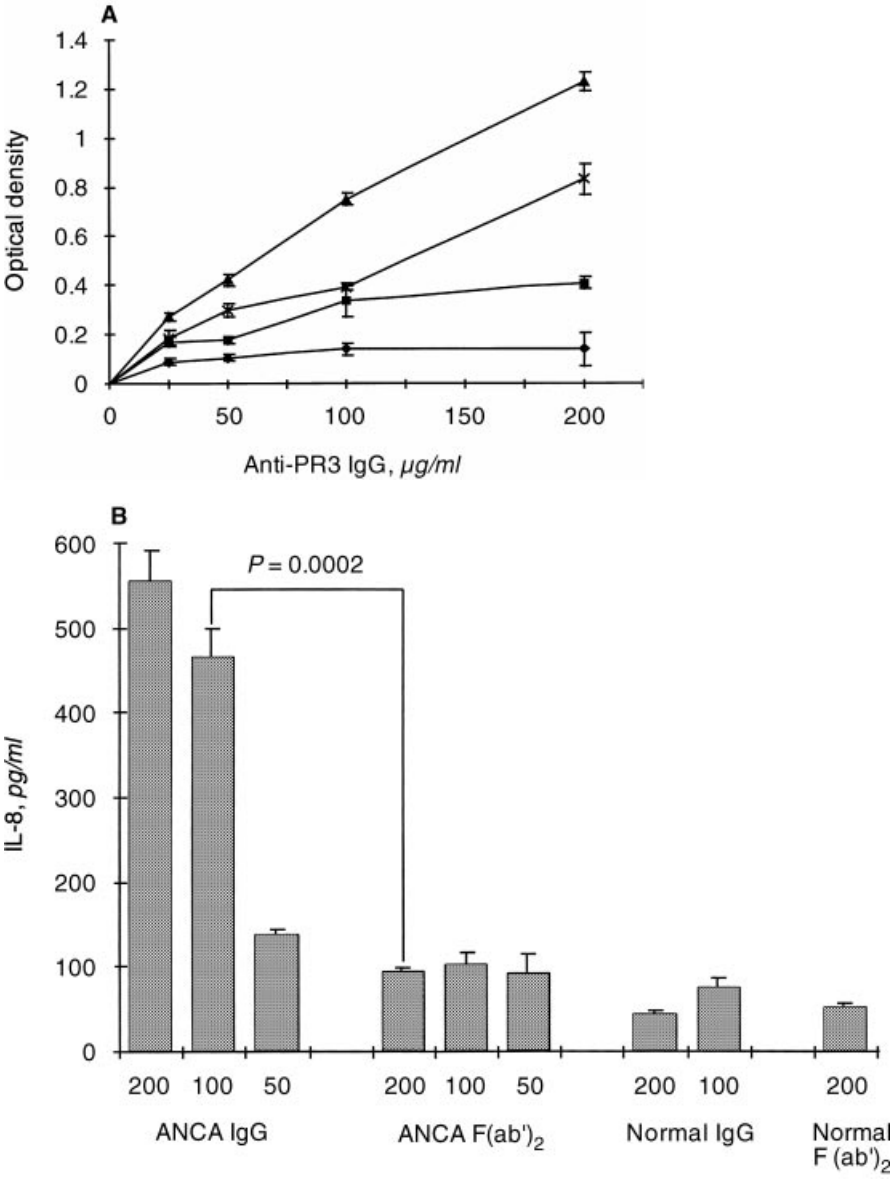

Fig. 4. (*A*) Binding activity of anti-PR3 ANCA (▲), anti-PR3 F(ab′)$_2$ (X), normal IgG (■) and normal IgG F(ab′)$_2$ (◆) against immobilized PR3. Results are shown as mean ± SEM of triplicates of a single experiment and indicate that 200 μg/ml anti-PR3 IgG F(ab′)$_2$ has equivalent binding activity to just over 100 μg/ml whole anti-PR3 IgG. (*B*) Stimulation of neutrophil IL-8 production by whole IgG and F(ab′)$_2$ preparations used in panel A. Data are shown as mean ± SEM of triplicates of a single experiment and demonstrates a major and significant difference in IL-8 production by 100 μg/ml whole anti-PR3-stimulated neutrophils compared with 200 μg/ml anti-PR3 F(ab′)$_2$ fragments, that is, preparations with an equivalent binding capacity. Human anti-MPO whole IgG and F(ab′)$_2$ fragments produced similar data.

tive oxygen radicals and cytokines [17, 18]. It is suggested that cytokine priming of neutrophils permits translocation of PR3 and MPO to the cell surface, where these molecules are accessible for binding by ANCAs. The ANCA antigen is ligated by the F(ab′)$_2$ component of the IgG molecule, which may then trigger neutrophil activation by binding to Fcγ receptors and activating downstream signaling pathways [30]. We reasoned that if ANCA-activated neutrophils also produced IL-8, then the production of IL-8 from ANCA-activated intravascular neutrophils might impede their transmigration toward extravascular IL-8, because intravascular IL-8 has previously been shown to cause sequestration of neutrophils within the microvasculature [4]. The effect of intravascular IL-8 is probably due to rapid and profound stimulation of F-actin polymerization with redistribution of neutrophil actin microfilaments and reorganization of the cytoskeletal architecture [31]. These changes in the neutrophil cytoskeleton lead to decreased neutrophil deformability and a physical frustration of the processes of trafficking.

In these studies, we demonstrate that ANCAs can stimulate production of IL-8 in a time- and dose-dependent manner. Furthermore, that blockage by IL-8 antibodies prevents this biologically active effect in chemotactic assays. In a separate assay system, this supernatant is a significant inhibitor of neutrophil trans-endothelial migration down an IL-8 gradient, suggesting that the autocrine effects of IL-8 by ANCA-activated neutrophils are to prevent transmigration.

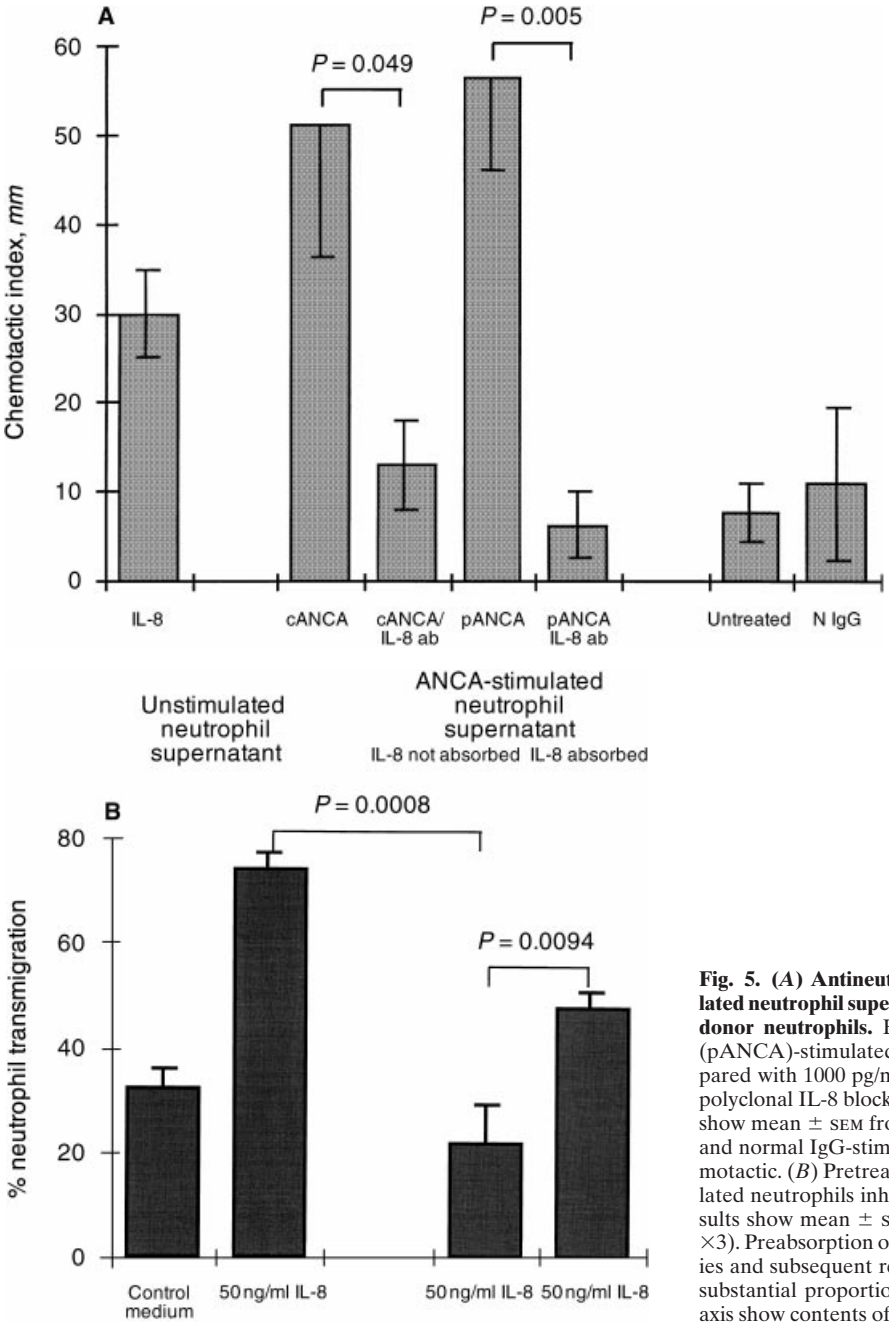

Fig. 5. (*A*) Antineutrophil cytoplasmic autoantibody (ANCA)-stimulated neutrophil supernatant is chemotactically active for normal human donor neutrophils. Both anti-PR3 ANCA (cANCA) and anti-MPO (pANCA)-stimulated neutrophil supernatant are effective when compared with 1000 pg/ml IL-8. This effect is blocked by the addition of a polyclonal IL-8 blocking antibody (IL-8 ab) to the supernatant. Results show mean ± SEM from triplicates of single experiments. Unstimulated and normal IgG-stimulated neutrophil supernatant was minimally chemotactic. (*B*) Pretreatment of neutrophils with supernatant from stimulated neutrophils inhibits transmigration across an EC monolayer. Results show mean ± SEM in triplicates of a single experiment (repeated ×3). Preabsorption of neutrophil supernatant IL-8 by anti-IL-8 antibodies and subsequent removal of complexes on protein G indicate that a substantial proportion of this effect is IL-8 attributable. Labels on *x*-axis show contents of medium below the transwell (endothelial) inserts.

The kinetics of expression of IL-8 by ANCA-stimulated neutrophils are different to the time scale of trafficking of a leukocyte at an inflammatory site. Intravital studies indicate that this process occurs rapidly, whereas our analyses show delayed ANCA-stimulated neutrophil IL-8 induction. This does not preclude IL-8 inhibition of neutrophil trafficking, as previous studies have indicated elevated circulating levels of IL-8 in small vessel vasculitis [32].These elevated levels may, in part, be secondary to ANCA ligation of neutrophils within the intravascular compartment, a process that may occur within the glomerular capillary vasculature or at other intravascular sites. These elevated levels of IL-8 may become particularly important within the glomerular capillaries, as other factors such as flow, capillary diameter, and endothelial heterogeneity may predispose to neutrophil activation and retention at this site. Furthermore, ANCA itself can rapidly decrease neutrophil deformability and may play an important role in promoting sequestration but inhibiting transmigration *in vivo* [33]. In addition, ultrastructural studies show that the earliest changes of ANCA-associated vasculitis affect the vascular endothelium it-

self, with swelling, necrosis, and de-adherence [34]. Thus, thrombosis within capillary loops and decreased deformability by ANCAs may lead to the intravascular retention of neutrophils. Subsequent sequestration of IL-8 by proteoglycans on de-adhered endothelium within the disrupted capillary may prevent trafficking. Intravascular IL-8 produced by ANCA-ligated neutrophils at this site may then exert paracrine effects on incoming neutrophils and further inhibit transmigration to extravascular IL-8. Such capillary retention of activated neutrophils is likely to sustain endothelial cell bystander damage and the vasculitic lesion. The early presence of non-migratory neutrophils within lesions [10] indicates that these cells have a central role in initiating focal segmental necrosis (the early vasculitic glomerular lesion). Later, other mechanisms are likely to perpetuate and extend the lesion to full-blown crescentic glomerulonephritis.

If the collective mechanisms we discussed earlier here do initiate early endothelial damage, we believe that we have identified a central role for the ANCA-neutrophil-IL-8 network in this process. Early therapeutic targeting to interrupt this network would be a logical extension to these studies.

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